THE EFFECTS OF THE NATURAL PRODUCT PARTHENIN ON THE DEVELOPMENT OF *PLASMODIUM* IN THE ANOPHELINE MOSQUITO

by

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Abstract

Background: Parthenin is a natural product with a wide array of biological effects that is derived from the invasive and damaging weed, *Parthenium hysterophorus*. Parthenin is closely related in structure to artemisinin, which is one of the most important antimalarial drugs in the world today. Both compounds are sesquiterpene lactones and research shows that parthenin also has an effect on *Plasmodium* blood stage development. This study did not evaluate parthenin as a potential therapeutic against blood stages but rather examined the effect of the compound on the transmissible stages of the parasite within the mosquito to gain potential mechanistic insight. This research could help identify a possible mode of action of parthenin, which may be different from artemisinin; and therefore pave the way for the development compounds more suitable as drugs that would be effective against artemisinin-resistant parasites.

Methods: We analyzed the developmental stages of *Plasmodium* in reverse order in the mosquito. First, serial dilutions of parthenin were included in *Plasmodium falciparum* gametocytemic blood and fed to *Anopheles gambiae* mosquitoes to evaluate the effect of the compound on oocyst infection intensity. Ookinete development in the presence of parthenin was subsequently evaluated by immunofluorescence as well as by imaging flow cytometry using a cultureable strain of *Plasmodium berghei*. Male microgamete exflagellation following 16 min incubation with parthenin was quantified by manual counting under phase contrast microscopy. Finally, to determine if parthenin can act on quiescent stage V gametocytes, the parasites were exposed to parthenin for 24 hours, and fed to mosquitoes without the compound present in the blood meal.

Results: Including parthenin in the blood meal resulted in a dramatic decrease in *P.*

falciparum oocyst numbers and was statistically significant at 100, 50 and 25 µg/mL. Ookinete formation in the midgut was also inhibited in a similar fashion at similar concentrations and imaging flow cytometry also demonstrated the absence of mature, *in vitro* cultured *P. berghei* ookinetes. As suspected, microgametocyte exflagellation was significantly inhibited by the compound, which directly impacts the number of ookinete produced. *P. falciparum* stage V gametocytes were rendered inactive after 24 hours of incubation with parthenin at 1 µg/mL as evident by the complete absence of exflagellation and oocyst formation despite the absence of the compound in the gametocytemic blood meal.

Conclusion: Parthenin was shown to have significant effects on all tested developmental stages of the parasite including gametocytes, male gametes and ookinetes. The broad inhibitory properties of parthenin on all the evaluated parasite stages may suggest different mechanisms of action between parthenin and artemisinin.

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Secondary Reader: Douglas E. Norris, MS, PhD

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Background

An estimated 198 million cases of malaria and 584,000 deaths, mostly among children, were reported in 2013 [1]. However, as a result of concerted control efforts since 2000, including anti-malarial drugs, mortality has declined by 47% and the prevalence in children ages 2-10 has dropped by 48%; resulting in an estimated 4.3 million lives saved [1]. Artemisinin, delivered with a partnered drug, is one of the fastest acting antimalarial therapies available [2, 3]. However, antimalarial resistance in *Plasmodium falciparum* has been described recently in Southeast Asia [4]. This discovery emphasizes the need for novel compounds that would be effective against resistant strains, presumably by acting on the parasite differently from artemisinin. Artemisinin was originally isolated from *Artemisia annua,* or sweet wormwood, a member of the Asteraceae family. We hypothesize that natural product compounds from other related family members may retain potent activities against *Plasmodium* while targeting different biological pathways.

Parthenin is a sesquiterpene lactone derived from *Parthenium hysterophorus*; which is an invasive, flowering annual weed in the Asteraceae family that grows to 2 meters in height with small white flowers and is spread throughout much of the world [5]. *P. hysterophorus* has been described as one of the world's most devastating weeds because of the millions of dollars in damages it causes yearly from diminished agricultural productivity [6, 7]. In addition to its detrimental effects on agriculture, the weed also causes allergic contact dermatitis. The overall negative impact of the plant on agriculture and human health has led to numerous control efforts including herbicides, burning the plant and biological control with numerous arthropod species [5, 8, 9]. Conversely, there is also an interest in the possible beneficial properties of the plant and its natural compounds. Parthenin is one

the best studied natural compounds derived from *P. hysterophorus* and is the main irritable component found throughout the plant tissue. Parthenin is secreted to inhibit growth of nearby plants and is the main agent responsible for allergic contact dermatitis [10-13]. Further studies on parthenin have revealed that it has a wide array of potentially beneficial biological effects and has shown activity as a potential herbicide [12, 14, 15] pesticide [16, 17] anti-parasitic [18, 19] and anti-cancer compound [20-23]. Parthenin may act through a variety of mechanisms including directly damaging DNA, inhibiting oxidative phosphorylation, inducing formation of nitric oxide and promoting apoptotic signaling [20, 21, 24-27]. General cytotoxicity of parthenin has been a concern but its activity against different cell lines ranges greatly, the IC50 of parthenin against Jurkat cells is .061 μM and 594 μM against HeLa cells [28]. Less toxic parthenin derivatives have also been made and show the potential promise of parthenin derivatives even if pure parthenin is cytotoxic against a number of cell lines [19, 21, 28].

Parthenin is a sesquiterpene lactone, a class of compounds that also includes the potent anti-malarial drug artemisinin. Artemisinin is one of a number of compounds that save lives by effectively treating the asexual blood stage form of *Plasmodium*. However, medicines that treat not only the asexual stages but also the transmissible forms of the parasite will be essential if we are to prevent the spread of new cases of malaria and bring about the eventual eradication of the parasite [29]. Fully mature, gametocytes (0.1-2% of the total parasite biomass), are the sexual, transmissible stages of *Plasmodium* [30]; the only stages that can continue the parasite life cycle in the mosquito following uptake in a blood meal. As the gametocytes enter the mosquito, the male and female gametes emerge from the red blood cell and the male gametes undergo 3 rounds of rapid DNA

replication after which the male gamete sends forth its DNA in flagellated packets in a process called exflagellation. After the female macrogamete is fertilized, the developing zygote will eventually give rise to an elongated mature ookinete. After approximately 24-36 hours the ookinete will cross the peritrophic matrix and glide along the brush border microvilli of the midgut epithelial cells before invading the mosquito's midgut epithelium [31]. The ookinete penetrates the epithelium to the basal lamina and begins developing into an oocyst. After 10-20 days of development (depending on *Plasmodium* species) these oocysts will rupture, releasing thousands of sporozoites into the hemocoel, which in turn attach to and invade the salivary glands where they await their next opportunity to infect a new host during a subsequent blood meal.

Understanding the biology of these parasite stages is important because very few antimalarial treatments that affect the asexual blood stages have an effect on the parasite stages involved in transmission. Artemisinin and its derivatives are one of the few compounds that show some efficacy against gametocytes, but its effects appear to be restricted to immature gametocytes, not the circulating mature stage V gametocytes [32-34]. Parthenin's relatedness to artemisinin and its demonstrated activity against intracellular blood stages raises the possibility of an extended efficacy against the transmissible stages [19], most of which are extracellular, thereby increasing potential exposure to the compound.

Provocative evidence of parthenin's potential transmission blocking activity comes from studies of the sugar feeding preferences of *An. gambiae* in Kenya. It was observed that *An. gambiae* preferentially nectar feed on *P. hysterophorus* [35]. It was also shown that mosquitoes feeding on this plant had shortened lifespans but mosquitoes infected with

Plasmodium feed more frequently on this plant [36, 37]. Most importantly, they observed that when mosquitoes were provided *P. hysterophorus* clippings before and after feeding on gametocytemic blood, these mosquitoes had lower oocyst burdens compared to mosquitoes that were not exposed to the plant (unpublished data). Based on these initial studies, we hypothesized that parthenin will inhibit *Plasmodium* extracellular development in the mosquito. Importantly, although artemisinin and parthenin are derived from members of the same family, parthenin does not contain the endoperoxide bridge that is essential for artemisinin's activity. This endoperoxide bridge is hypothesized to act as a prodrug requiring cleavage of bridge to induce reactive oxygen species [19, 38]. We would therefore predict that comparable activities against blood and mosquito stages are a result of different modes of action of the two compounds, which is important given the observed parasite resistance to artemisinin combined therapy. Furthermore, given the dynamic nature of the transition of gametocytes to extracellular ookinetes and oocysts, we hypothesize that parthenin will have a significant effect on parasite development in the mosquito. Here, we report on the potent transmission blocking activity of parthenin against *P. falciparum* in *An. gambiae* as well as its uptake by stage V gametocytes and its specific effect on male microgamete activation.

Methods

Parasite strains

P. falciparum NF54 was the main parasite used for the majority of this study because *P. falciparum* is the deadliest strain of *Plasmodium* and clinically relevant throughout much of Sub-Saharan Africa. However, *P. falciparum* mosquito stages are difficult to culture *in vitro* and would limit the scope of this study to only one species. Therefore a *P. berghei*

CTRP-GFP strain was used to further investigate if parthenin would affect other *Plasmodium* species as well as provide a suitable strain for *in vitro* culture. We are confident that the results seen in either strain are comparable to one another and should not adversely affect the results of this study.

Preparation of parthenin stocks

Parthenin was isolated from whole plant tissue using methanol extraction and purified by HPLC [39]. The extract was dissolved into acetone and then diluted 1:10 into water to give a stock solution of 5 mg/mL. Parthenin purity was reconfirmed by HPLC after being dissolved into the acetone water mixture (Supplemental F1).

Mosquito rearing and Standard Membrane Feeding Assay (SMFA)

An. gambiae KEELE mosquitoes were maintained at the Johns Hopkins Malaria Research Institute Insectary. Mosquitoes were kept at 26 °C and 70% humidity with 12:12 hour light-dark cycles and sustained on cotton soaked in 10% sucrose.

P. falciparum NF54 gametocytes were cultured in RPMI 1640 containing HEPES and glutamine and supplemented with 10% human serum and hypoxanthine. *P. falciparum* NF54 strain was diluted to 0.5% mixed stage asexual parasites and 4% hematocrit in complete culture medium in six well plates. These plates were transferred to a 37 °C incubator at day 15 and a microaerophilic environment was created using a desiccator candle jar. Media was exchanged daily from day 1 to day 17 and standard membrane feeding assays (SMFA) were done on days 15-18 post-culture initiation.

Approximately 50 female *An. gambiae* mosquitoes were distributed into pint sized cups and starved of sugar and water for ~12 hours prior to feeding. *P. falciparum* (NF54) day 15-18 gametocyte cultures were pelleted and diluted to 0.25% or 0.1% gametocytemia with human blood at 50% hematocrit. Blood was washed with RPMI media and brought to 50% hematocrit with normal AB serum. Gametocytemic blood was kept at 37 °C until feeding. Parthenin was diluted to appropriate concentrations in 20 µL total volume + 2 µL of 10x PBS. Each parthenin dilution (22 µL total volume) was added to 200 µL of gametocytemic blood. The gametocytemic blood was placed inside of water-jacketed membrane feeders at 37 °C and mosquitoes were allowed to feed for 60 minutes. After blood feeding, non-blood fed mosquitoes were removed from each treatment and the mosquitoes were kept at 26°C and 70% humidity for 8 days prior to midgut dissections.

Midguts were dissected 8 days post-feeding and stained with 0.2% Mercurochrome dye in water for 20 minutes. Midguts were placed on a slide with a drop of PBS and oocysts were counted by brightfield microscopy at 200x total magnification. Where appropriate, a generalized linear mixed model (GLMM) or a nonparametric test (Mann-Whitney U test and Kruskall-Wallis one-way analysis of variance with subsequent Dunn multiple-comparison tests) were used to evaluate differences in oocyst intensity between control and treatment groups. Significance level, $\alpha=0.05$.

Immunofluorescence Assay

Mosquito midguts were dissected 22 hours post infectious blood feed. The blood meal was separated from the midgut by nicking the bottom of the midgut and using the surface tension of the PBS to collect the blood meal contents. The midgut was discarded and the individual blood meals were homogenized and fixed in 100 µL of 4% paraformaldehyde.

Samples were centrifuged at 3,000 RPM and washed with PBS and were centrifuged again and the PBS removed and the sample was blocked with 3% Bovine Serum Albumin for at least 30 minutes. Samples were washed 3x with PBS and probed with α-Pfs25 antibody (1:1000) for 1 hour at room temperature. Then blood meal content was washed 3x in PBS and then probed with an Alexa Flour 488-conjugated α-mouse secondary antibody (Invitrogen, Carlsbad, CA). Samples were washed 3x in PBS and then 10 μL was spotted onto a glass microscope slide. After drying, a drop of Prolong gold antifade reagent with DAPI (Invitrogen) was added before covering with a glass coverslip. Slides were imaged at 1000x total magnification using a Nikon 90i digital microscope and velocity imaging software.

***P. berghei* ookinete production and purification**

Donor mice were infected with a frozen stock of a CTRP-GFP strain of *P. berghei*. After 7-10 days another set of mice were injected with 200 μL of 0.63% phenylhydrazine in PBS to induce reticulocytosis. After 3 days, phenylhydrazine treated mice were infected with blood from a donor mouse that was at least ≥15% parasitemia. At 3 days post-infection, mice were given 15 mg/L sulfadiazine in their drinking water to reduce the number of asexual parasites. After 24 hours the number of exflagellation centers was measured for each mouse, and those mice with parasitized blood presenting with ≥15 exflagellation centers/20x field were sacrificed and blood collected by heart puncture using a heparinized needle. Blood was placed directly into complete ookinete culture medium and rocked gently for 48 hours at 19°C.

Incomplete ookinete culture media is prepared with 16.4 g RPMI with HEPES and

L-glutamine (Sigma-Aldrich) in 1 liter, 0.2% weight per volume $NaHC0_3$, 0.05% weight per volume hypoxanthine (Sigma-Aldrich), 100 µM Xanthurenic acid (XA, Sigma-Aldrich), 50 units/mL penicillin, 50 µg/mL streptomycin (100x penicillin and streptomycin, Invitrogen), pH 7.4. The medium is filter-sterilized and can be stored at 4 °Celsius (up to 6 months). Complete media is made prior to use by supplementing with heat inactivated FBS to 20% FBS by volume.

After 48 hours, ookinetes were enriched using a MidiMACs quadromacs magnet column (Miltenyi Biotec) using LS columns equilibrated with 5 mL of incomplete ookinete media. The blood from the 48 hour ookinete culture was run through the LS column twice. The column was washed 3x with 5 mL of complete ookinete media. The LS column was then removed from the magnet and the ookinetes were eluted with 3 mL of complete ookinete media. Ookinetes were spun down at 3,500 RPM for 3 minutes and extra media removed to concentrate the samples as needed for flow cytometry.

Quantification of ookinetes by flow cytometry

P. berghei CTRP-GFP ookinetes were prepared as described, in 96 well plates, with each well containing 100 µL of complete ookinete media, 10 µL of parthenin in acetone at concentrations of 100, 50, 25, 12.5 and 6.25 µg/mL and 5 µL of gametocytemic blood from a single mouse. Samples were run in sets of four and pooled during purification. Samples were spun down at 3,500 RPM to concentrate to 30 µL of total volume in complete ookinete media. After enrichment, approximately 1-8% of the cells were mature ookinetes.

Samples were analyzed on an Amnis Imagestream^X Mark II flow cytometer with high

resolution imaging capabilities. Individual images of each event were recorded. The events were gated based on brightfield area and GFP intensity. Images were taken at 60x magnification and run at high speed with a 7 µm core size. To increase the speed of the analysis and overcome the throughput limitation of the Imagestream, only an equivalent portion of each sample was analyzed and the total number of ookinetes per sample was estimated based on the proportion analyzed.

Data analysis was performed using the IDEAS version 6.0 software, with 1×10^5 to 1×10^6 as the selected range of GFP intensities that are indicative of the fertilized parasites based on a visual confirmation of the live images as the parasites passed through the laser. The parasites were also gated based on brightfield area to exclude events that would be too small or large to be parasites. Each event within the gate was later individually verified as a parasite or another cell type.

Quantification of exflagellation centers

4 µL of *P. falciparum* gametocytemic blood was pipetted on a glass slide and incubated at room temperature for 16 minutes. After 16 minutes, exflagellation centers were counted across 10 random fields at 200x total magnification on a compound microscope using phase contrast microscopy. Counts for each sample were performed in triplicate at 6.25-100 µg/mL parthenin.

Assessment of the functional activity of stage V gametocytes

Gametocytes were cultured as described previously except that on day 15-16, gametocytes were exposed to 100, 10 and 1 µg/mL parthenin for 24 hours. The control

gametocytes were exposed to the water acetone mixture over the 24 hour time period. After 24 hours, the treated media was washed out and replaced with fresh media; after another 24 hours of exposure to the fresh media, the gametocytes were used in a SMFA. Midguts were dissected and oocysts counted as described as a measurement of gametocyte viability and infectivity to mosquitoes.

Results and Discussion

Exposing *P. falciparum* gametocytes to parthenin in the blood meal results in a significant decrease in oocyst intensity in the mosquito

Addition of parthenin to gametocytemic blood at 100, 50, 25, 12.5 and 6.25 µg/mL prior to ingestion by the mosquitoes was shown to have a significant impact on oocyst intensity at 100, 50 and 25 µg/mL in all three replicates (Figure 1 A and Supplementary Figure 2 A and B). Even at the lowest concentration of 6.25 µg/mL, median oocyst counts decreased by 40-80%, but oocyst prevalence remained high at 88-91% (Figure 1B). The water acetone mixture used in the control feeds was previously observed to have negligible impact on the number of oocysts (data not shown).

Inclusion of parthenin in the blood meal effectively shows the transmission blocking activity of parthenin; however, parthenin may have acted on the parasite at any of the developmental stages from early gametogenesis to oocyst formation. To determine the stage affected by parthenin we examined each developmental step preceding oocyst formation.

Parthenin decreases the numbers of zygotes and ookinetes in *P. falciparum in vivo* and *P. berghei in vitro*

To explore if there is any effect of parthenin on ookinete development, mosquitoes were fed *P. falciparum* NF54 gametocytes with a high concentration of parthenin (500 µg/mL, a concentration at which oocysts were never observed), dissected 22 hours post blood feed and checked for the presence or absence of ookinetes by IFA. At this concentration, none of the treated mosquitoes had any ookinetes in the midgut while all the control mosquitoes were positive for ookinetes (Supplemental Figure 3).

Due to the difficulties associated with obtaining accurate counts of total ookinetes *in vivo*, a *P. berghei* line expressing GFP under the control of the CTRP promoter was used in an ookinete in vitro culture [40].This approach allow for the precise titration of parthenin, under controlled culture conditions and quantification by flow cytometry.

The Amnis Imagestream^{X} MKII system quantifies and captures the image of each GFP positive event (parasite), which represents only fertilized female macrogametes, zygotes, retorts and mature ookinetes. There was a decrease in speed at which the instrument could calculate total events but the ability to match an image with each event (at 600x magnification) was significant, as this allowed manual confirmation of the quantified parasites (Figure 2A). A red blood cell could be easily distinguished from a zygote or an ookinete (Figure 2A) and parasites obscured by other cellular debris were also verified (Supplemental Figure 4).

The manually verified counts of total zygotes and ookinetes demonstrated that parthenin

in the blood meal decreases the total number of parasites prior to midgut cell invasion and the development of oocysts (Figure 2 B-F); corroborating our initial observations (Figure 1). Overall 58% of the events were verified as parasites (Figure 2F). Of the three replicates, we determined that replicate 2 had an inordinate number of cellular debris of which only 46% of the positive events were intact parasites. On the other hand, the proportion of intact parasites in replicates 1 and 3 were 93% and 84% positive, respectively. Of all the parasites imaged, approximately 90% were round zygotes and 10% were fully developed ookinetes with a small proportion of the parasites in the intermediate retort form (Figure 2F). It could not be ascertained definitively whether or not the observed ookinetes were viable, or functional, as the parasites were not sorted; however, we assume this to be the case given the normal ookinete morphology (Figure 2A) and the observed development of oocysts present *in vivo* with matched concentrations of parthenin (Figure 1).

Parthenin decreases exflagellation

The observed decrease in ookinetes with increasing concentrations of parthenin suggests that parthenin's impact may precede fertilization. Exflagellation may be vulnerable to the hypothesized effects of parthenin because parthenin has been shown to damage DNA as well as quickly replicating cancerous cell lines [20, 24-26]. This makes male gametes a potentially sensitive target because microgametogenesis and exflagellation involve intense DNA replication and partitioning to flagella. As suspected, we observed a significant decrease in the number of exflagellation centers with increasing parthenin concentrations from 6.25 μg/mL to 100 μg/mL (Figure 3).

This reduction of exflagellation illustrates the speed at which parthenin acts on the parasite, as exflagellation centers were measured after a 16 minute exposure to compound. The effect on exflagellation and speed at which the compound acts may give some insight into possible mechanisms of action, if the detected decrease in exflagellation centers is a result of the main mechanism of action of parthenin. Damage to the DNA or induction of reactive oxygen species during this time of intense DNA replication and cellular partitioning may be responsible for the observed effects. One mechanism of action that loses support in this assay as a major contributor of parthenin's activity is that parthenin inhibits oxidative phosphorylation [27]. Male gametes appear to lack mitochondria and have been shown to rely on glycolysis quite heavily for exflagellation [41, 42]. Further focus on the inhibition of this specific parasite stage may be fruitful for trying to unravel potential mechanisms of action due to the robust activity during microgametogenesis.

Parthenin inactivates stage V gametocytes

Mature *P. falciparum* stage V gametocytes that circulate in the peripheral blood have proven difficult to eliminate by standard antimalarial drugs that are effective at killing asexual stages [34, 43]. Artemisinin is active against early gametocyte developmental stages, but not mature forms [32-34]. However, we wanted to know if parthenin can inactivate gametocytes in vitro without having to be ingested by the mosquito. We incubated stage V gametocytes with parthenin for 24 hours, washed the compound and incubated the parasites in normal culture medium prior to feeding 24 hours later. Our study suggests that parthenin is taken up by gametocytes and prevents exflagellation 24 hours post-exposure which leads to a lack of oocysts 8 days later (Figure 4). Testing stage V gametocytes was relevant for this study because it is the stage that bridges the gap

between the human and mosquito and while parthenin may have an effect on earlier gametocyte stages (I-IV), it is outside the focus of this study on parthenin's effect on the mosquito developmental stages of *Plasmodium*.

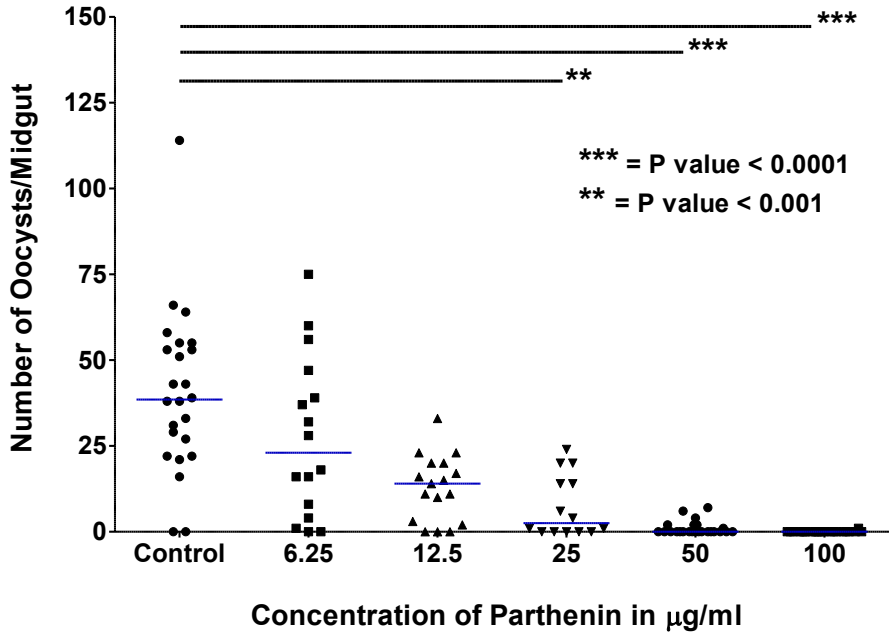
Conclusion

Parthenin has demonstrated an ability to inhibit each of the developmental stages of *Plasmodium* against which it was tested. This broad activity seems promising for parthenin as a potential therapeutic, and parthenin has been used as a medicinal herb, but its broad range of cytotoxic effects has thus far hindered its development as a therapeutic [26, 39, 44, 45]. However, previous studies have shown that chemical derivatives of parthenin and closely related compounds have shown similar inhibitory profiles with less general cytotoxicity; this greater specificity has allowed these compounds to reach clinical trials [19, 20, 22, 46]. Notwithstanding the cytotoxicity, the demonstration of the compound's antimalarial properties make it a valuable tool for investigating mechanisms underlying other parthenin derivatives or sesquiterpene lactones that may retain antimalarial activity but have a better safety profile. Studies like this become increasingly important as artemisinin resistance continues to grow.

In conclusion, parthenin has been demonstrated as a compound with activity against multiple mosquito stages of the *Plasmodium* parasite. Further studies on parthenin's mode of action could lead to the discovery of vulnerable *Plasmodium* pathways that are independent of artemisinin's proposed mechanism of action.

Figures

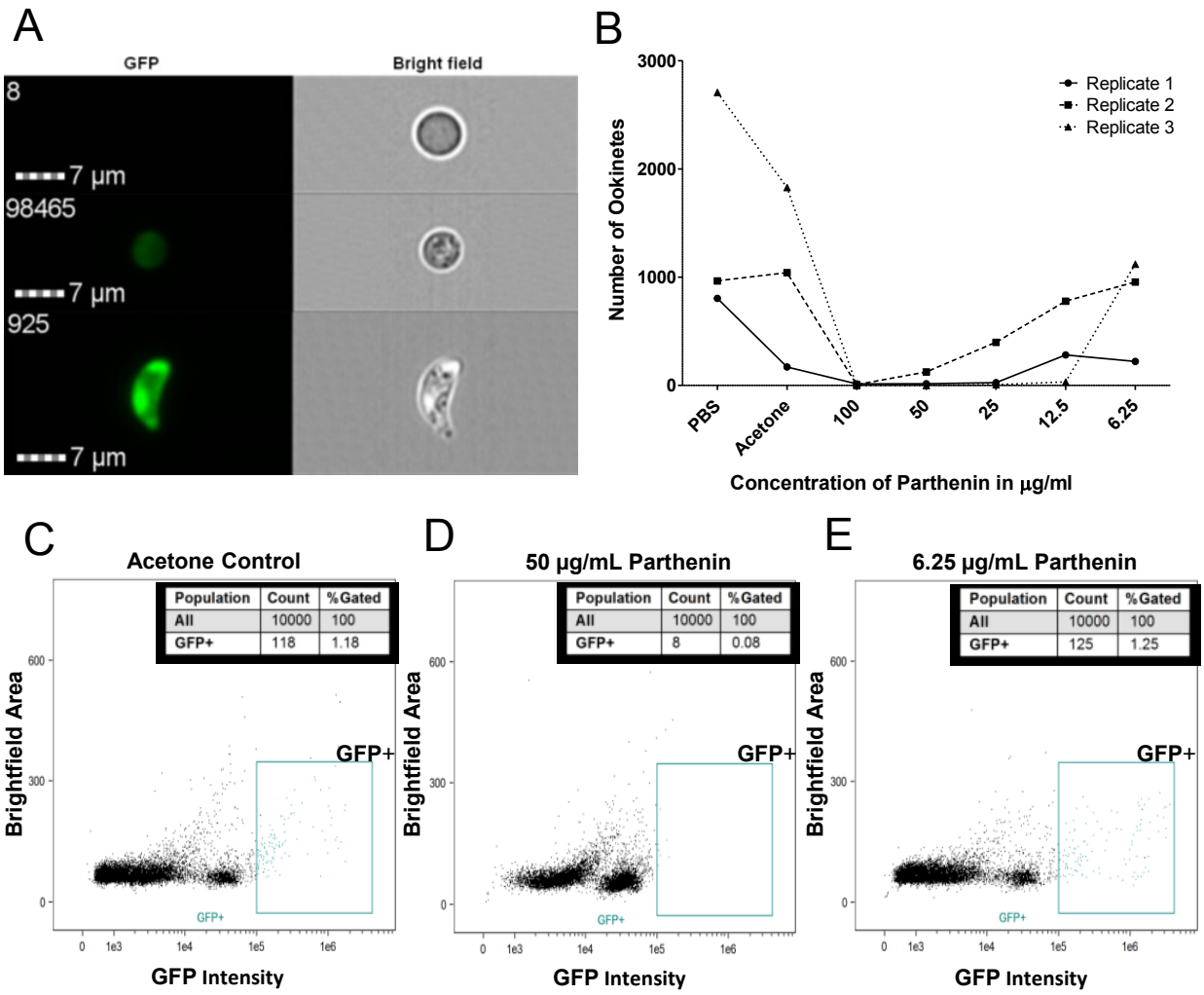
A



B

		Control	6.25 µg/ml	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml
1	Sample Size	14	18	32	20	26	24
	Median	77	15	1	0	0	0
	% Inhibition	NA	80.52	98.7	100	100	100
	Prevalence	93.33	91.67	72.22	50	0	0
2	Sample Size	24	16	17	14	24	22
	Median	38.5	23	14	2.5	0	0
	% Inhibition	NA	40.26	63.64	93.51	100	100
	Prevalence	92	88.24	83.33	60	28	4.35
3	Sample Size	37	45	33	39	40	30
	Median	23	10	5	0	0	0
	% Inhibition	NA	56.52	78.26	100	100	100
	Prevalence	89.47	91.11	90.91	22.5	4.88	10

Figure 1. Parthenin treatment of gametocytemic blood concurrent with blood feeding results in lower oocyst counts. **A.** Replicate (2) showing statistically significant decreases in *P. falciparum* oocyst counts at 100, 50 and 25 µg/ml. Statistical significance was determined using nonparametric statistical analyses (Mann-Whitney U test and Kruskall-Wallis one-way analysis of variance with subsequent Dunn multiple-comparison tests). **B.** Table summarizing the sample size, median oocyst number, % inhibition in median oocyst numbers and prevalence of infection for all three replicates (replicates 1 and 3 are shown in Supplementary Figure 2).



F

	Gated Parasites	Verified Parasites	% Verified	Total parasites in full volume	Total ookinetes in full volume	% Conversion
Replicate 1	327	304	92.97	1534	85	5.56
Replicate 2	2266	1033	45.59	4270	472	11.05
Replicate 3	648	544	83.95	5698	586	10.29
Average	3241	1881	58.04	11502	1143	9.94

Figure 2. Parthenin treatment inhibits *P. berghei* ookinete maturation. A. Example images from the Amnis Imagestream mkII depicting a red blood cell (top) zygote (middle) and ookinete (bottom); images like these were used for the manual verification of each GFP positive event. **B.** Graph depicting total number of zygotes and ookinetes at different concentrations that were identified by flow cytometry and individually verified. **C-E.** Representative flow cytometry results from replicate 3 depicting an example of a control, a higher concentration parthenin treatment (50 µg/mL) that contained no verified parasites and a low concentration treatment that contained a high amount of parasites (6.25 µg/mL). **F.** Table summarizing total numbers and percentages of gated and verified parasites and conversion rate of parasites from round zygotes to elongated ookinetes.

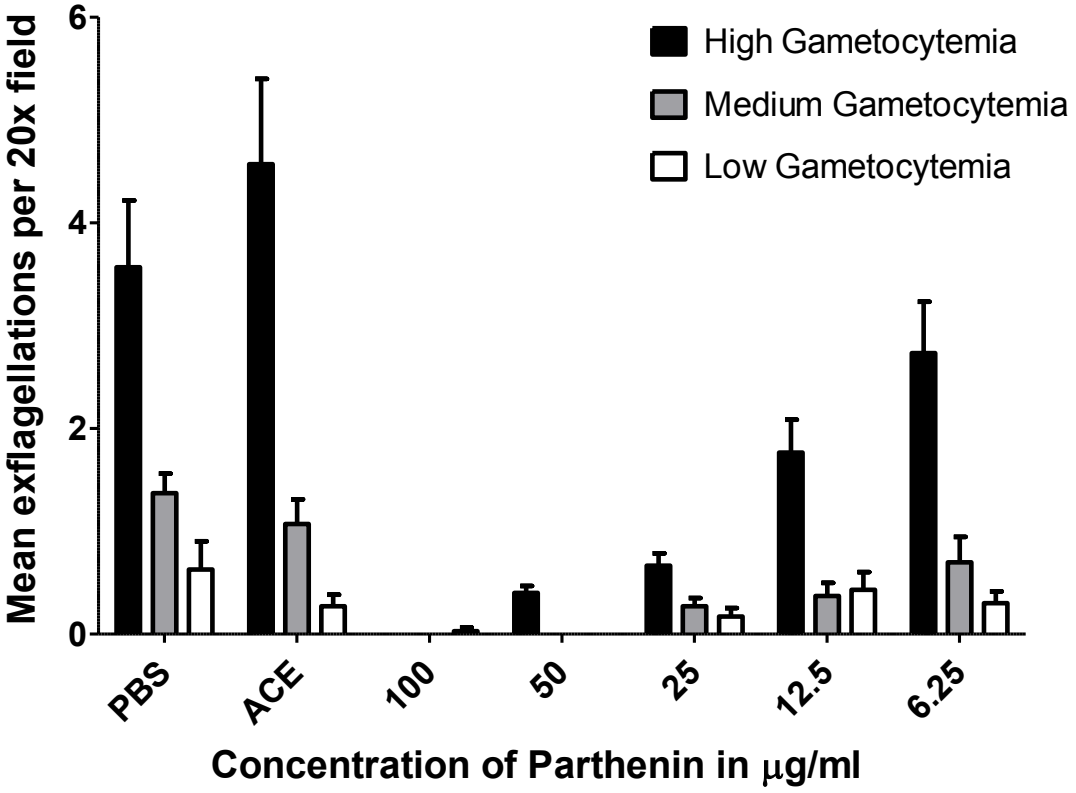
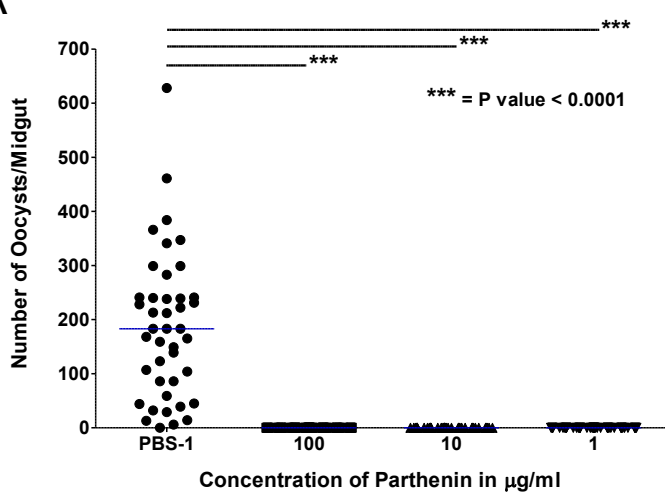


Figure 3. Parthenin decreases the number of exflagellation center events in *P. falciparum*. Each bar is the mean number of exflagellation centers seen across 30 fields at 200x total magnification 16 to 18 minutes post induction with error bars representing the standard error of the mean. High gametocytemia was 1% gametocytemia, medium gametocytemia was .25% and low gametocytemia was .1%. In all three replicates exflagellation counts were markedly decreased at higher concentrations and returned to higher levels at lower concentrations of parthenin.

A



B

	Control	100 µg/ml	10 µg/ml	1 µg/ml
Sample Size	42	47	48	57
Median	184	0	0	0
% Inhibition	NA	100	100	100
Prevalence	97.67	0	0	0

C

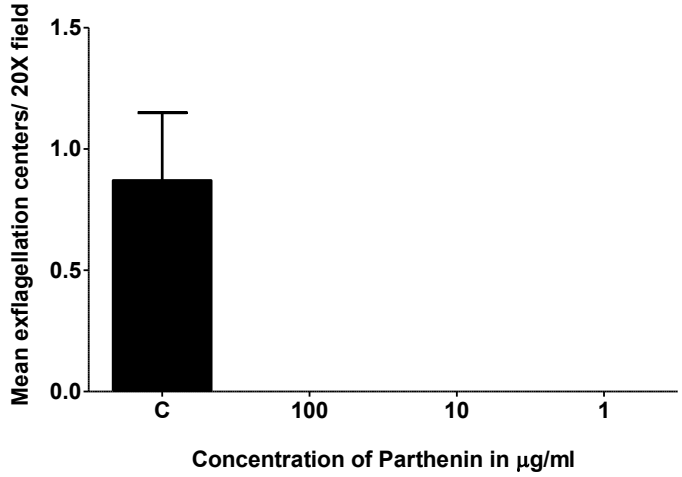
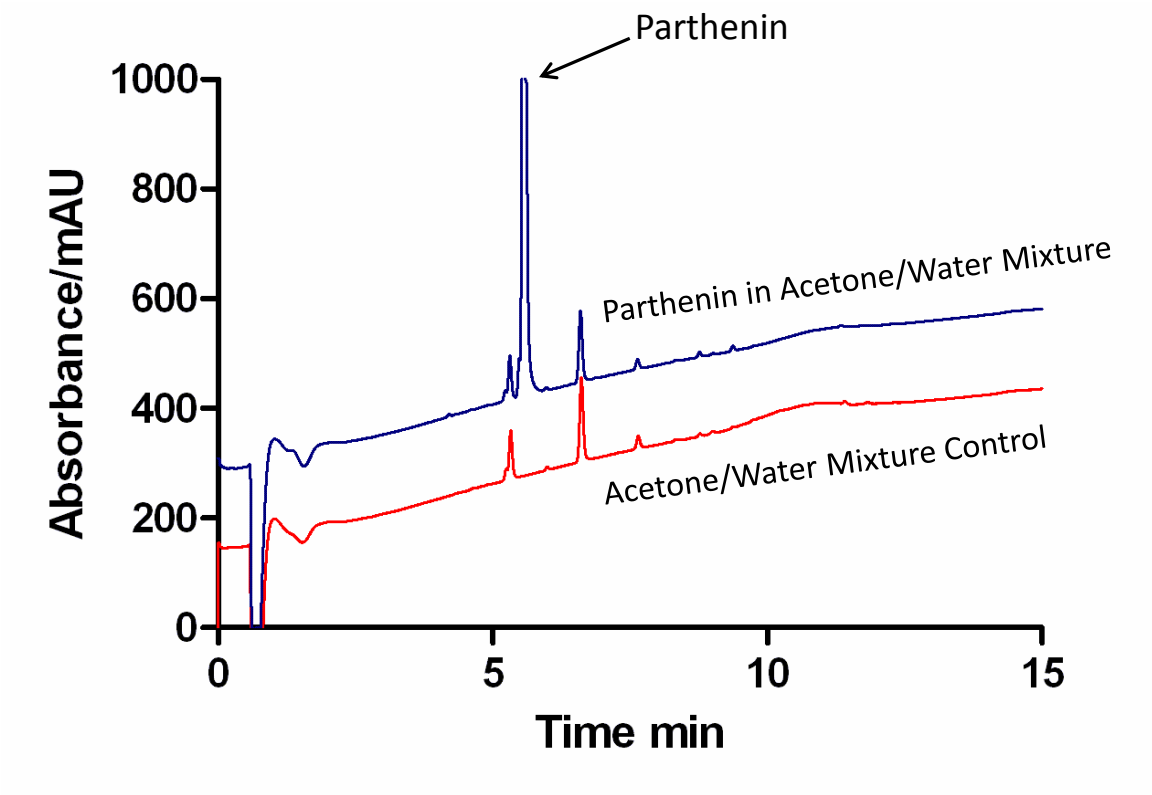
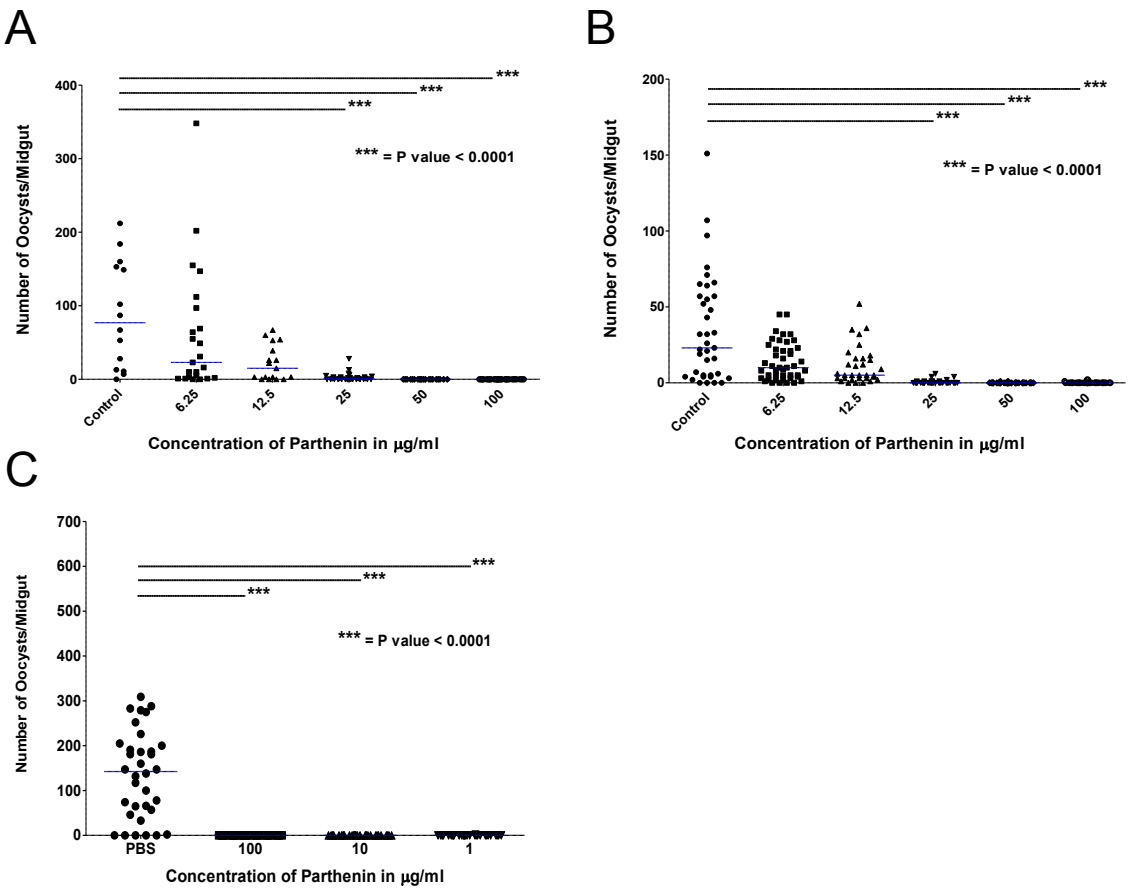


Figure 4. Co-incubation of parthenin with *P. falciparum* stage V gametocytes results in lack of exflagellation or oocysts. A. Oocyst intensity was reduced by 100 % at all three concentrations. **B.** Table showing the sample size, median oocyst number and % inhibition for oocyst counts from the graph in part A **C.** Mean exflagellation counts plus the standard error of the mean for the same gametocytemic blood used for the feed in part A; no exflagellation was observed at any of the concentrations.

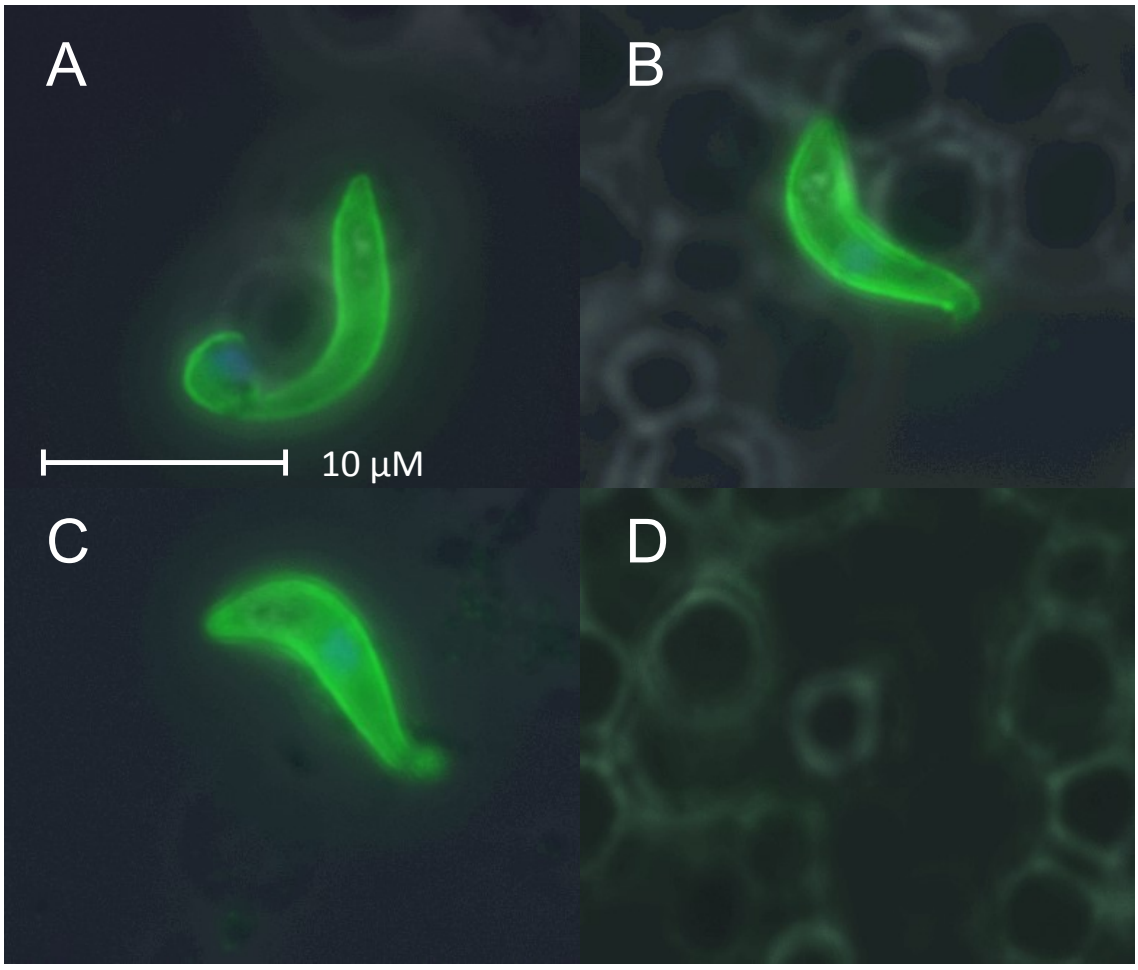
Supplemental Figures



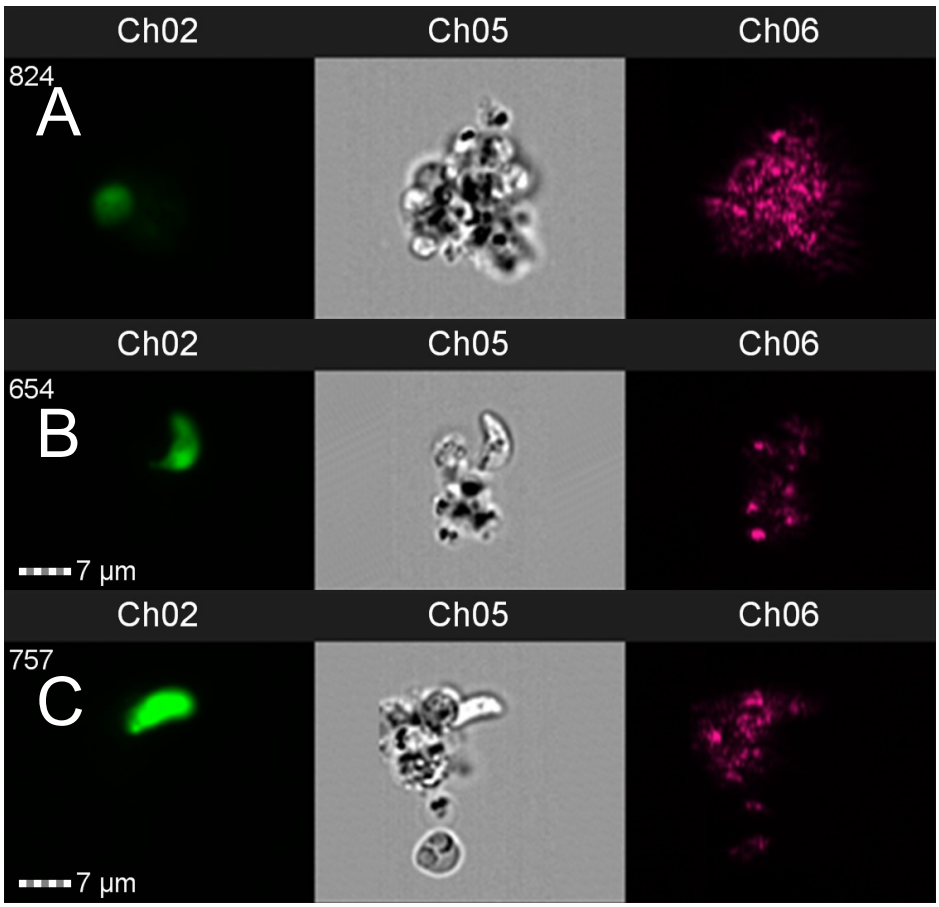
Supplementary Figure 1. Confirmation of parthenin purity by HPLC. The red line is the water/acetone mixture used to dissolve the parthenin. The blue line is the same mixture with the addition of parthenin. The strong peak near 6 minutes illustrates the purity of the parthenin.



Supplementary Figure 2. Replicates further illustrating the anti-parasite activity of parthenin. A. Biological Replicate 1 from Figure 1 illustrating the effect of parthenin on *P. falciparum* oocyst development when added to the blood meal immediately before feeding. **B.** Biological Replicate 3 from Figure 1 illustrating the effect of parthenin on oocyst development when added to the blood meal immediately before feeding**. C.** Technical Replicate 2 from Figure 4 showing the effect of parthenin when incubated with stage V gametocytes and then washed out before feeding.



Supplementary Figure 3. Lack of *P. falciparum* ookinetes *in vivo* at 500 µg/mL parthenin. Midgut contents were removed 22 hours post SMFA, fixed in 4% Paraformaldehyde, and probed with α-pfs25 antibody (1:1000) and probed again with Alexa Flour 488 α-mouse secondary antibody. **A-C.** Example ookinetes from the control groups that were all positive for ookinetes. **D.** An example image from the parthenin treated (500 µg/mL) mosquitoes showing that no ookinetes were present in any of the midguts, n=5 for both treatments.



Supplementary Figure 4. Sensitivity and precision of Amnis Imagestream^{X} Mark II instrument to decipher between GFP+ *P. berghei* parasites and cellular debris. Ch02 is GFP, Ch05 is brightfield image and Ch06 is side scatter. **A.** Round zygote obscured by a clump of cells **B-C.** Ookinetes that are also obscured by cellular debris but still detectable by GFP. These images illustrate the ability of the Imagestream to pick out parasites even when obscured by other cells or debris.

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CURRICULUM VITAE

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EDUCATION

Johns Hopkins University Bloomberg School of Public Health **2013-2015**
ScM, Masters of Science
Molecular Microbiology and Immunology
3.92 GPA

Brigham Young University **2008-2013**
B.S Bachelor of Science
Microbial Genetics
3.93 GPA *magna cum laude*

AWARDS

Johns Hopkins Tuition Fellowship **2014-2015**
ORCA grant **2013**
BYU Full tuition Scholarship **2008-2012**

RESEARCH EXPERIENCE

Dinglasan Lab **2014-2015**
-Trained and certified in *P. falciparum* and dengue virus infection protocol
-Trained in rearing anopheline mosquitoes, especially *Anopheles gambiae* and *An. farauti*
-Cultured *Drosophila* S2 cells for expressing and purifying recombinant *An. gambiae* protein
-Evaluated the anti-malarial activity of *Parthenium hysterophorous* plant extract
-Performed Immuno-Fluorescence Assays on developmental genes in the mosquito midgut
-Trained in handling and vaccinating mice especially in transmission blocking vaccine studies
-Performed ELISA's on mice serum from vaccine studies
-Submitted a research paper and a review paper as a first author

Dimopolous Lab **2013-2014**
-Raised transgenic *Aedes aegypti* mosquitoes
-Infected mosquitoes with DENV2 and calculated viral titer
-Performed qPCR to quantify mRNA levels of transgenes in study

Robison Lab **2012-2013**
-Prepared media and laboratory stocks of different bacterial strains

-Tested the inhibitory range of a thiopeptide producing strain of Staphylococcus epidermidis
-Helped purify large quantities of the thiopeptide and sequencing of the corresponding plasmid

RELATED EXPERIENCE

Brigham Young University
Teaching Assistant, Genetics **2012-2013**

Provided one on one help as well as large scale test reviews. I was approached by multiple students and offered private tutoring jobs to help in additional classes.

Utah Valley Regional Medical Center
Volunteer **2012**

Performed whatever tasks were necessary to best aid the hospital staff and became acquainted with basic hospital procedures.

Church of Jesus Christ of Latter Day Saints
Missionary, volunteer **2009-2011**

Spent two years engaged in daily service and learning the native languages as I also taught the beliefs of my religion. I served as a district and zone leader, supervising 10 and 25 missionaries respectively.

PUBLICATIONS AND PRESENTATIONS

Impact of the overexpression of *Aedes aegypti* innate immune proteins Hop and Dome on Dengue viral titers
Presented at the Johns Hopkins Research Seminar **2013**

Lipid rafts and *Plasmodium* invasion of the mosquito midgut epithelium
Submitted as first co-author to Parasites and Vectors **2014**

The effects of parthenin on the development of *Plasmodium* in the anopheline mosquito
Manuscript in preparation for submission as first author to the Malaria Journal **2015**

LANGUAGES

English-native speaker
Tagalog- fluent
American Sign Language- conversational

REFERENCES

References available upon request